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<table border="0" style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> <p>(21) International Application Number: PCT/SE00/00210</p> <p>(22) International Filing Date: 3 February 2000 (03.02.00)</p> <p>(30) Priority Data: 9900431-9 9 February 1999 (09.02.99) SE</p> <p>(71) Applicant (for all designated States except US): PROTEASE AB [SE/SE]; c/o Stenflo, Ärtholmsvägen 196, S-216 20 Malmö (SE).</p> <p>(72) Inventor; and</p> <p>(75) Inventor/Applicant (for US only): STENFLO, Johan [SE/SE]; Ärtholmsvägen 196, S-216 20 Malmö (SE).</p> <p>(74) Agent: AWAPATENT AB; Box 5117, S-200 71 Malmö (SE).</p> </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> <p>(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> </td> </tr> </table>			<p>(21) International Application Number: PCT/SE00/00210</p> <p>(22) International Filing Date: 3 February 2000 (03.02.00)</p> <p>(30) Priority Data: 9900431-9 9 February 1999 (09.02.99) SE</p> <p>(71) Applicant (for all designated States except US): PROTEASE AB [SE/SE]; c/o Stenflo, Ärtholmsvägen 196, S-216 20 Malmö (SE).</p> <p>(72) Inventor; and</p> <p>(75) Inventor/Applicant (for US only): STENFLO, Johan [SE/SE]; Ärtholmsvägen 196, S-216 20 Malmö (SE).</p> <p>(74) Agent: AWAPATENT AB; Box 5117, S-200 71 Malmö (SE).</p>	<p>(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
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<p>(54) Title: MONOCLONAL ANTIBODY</p>				
<p>(57) Abstract</p> <p>The present invention relates to a monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, a method for preparation of said monoclonal antibody, a method for monitoring the activity of systems involving protein C inhibitor and a method for diagnosis of <i>e.g.</i> venous thrombosis, wherein said monoclonal antibody is utilised. Said monoclonal antibody is suitable for monitoring the activity of systems involving protein C inhibitor, and it has specific affinity for both <i>i</i>) a complex between a serine proteinase and an inhibitor thereof, and <i>ii</i>) a cleaved and uncomplexed form of said inhibitor, but has substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or a derivative thereof having the same biological activity.</p>				

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MONOCLONAL ANTIBODYField of the Invention

The present invention relates to a monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, a method for preparation of said monoclonal antibody, a method for monitoring the activity of systems involving protein C inhibitor and a method for diagnosis of e.g. venous thrombosis, wherein said monoclonal antibody is utilised.

Background of the Invention

Protein C is a proenzyme to "activated protein C". Activated protein C, hereinafter denoted APC, is a serine proteinase, and its proenzyme protein C is a liver-synthesised glycoprotein having 461 amino acid residues and a molecular weight of approximately 62 kDa. Protein C carries two parts denoted the light and the heavy chain, respectively (formed by limited proteolysis of a single chain precursor). The light chain is glycosylated on its Asn 97 residue, whereas the heavy chain is glycosylated on its Asn 248, Asn 313 and Asn 329 residues (Asn 329 being glycosylated only in so-called "single chain protein C"). Of all the protein C in plasma, about 85% contains both the light and heavy chain, whereas about 15% consists of single chain protein C. There is no known difference in biological activity between these two forms of protein C.

The light chain of protein C contains an amino-terminal domain with nine γ -carboxyglutamic acid (Gla) residues, which have been formed by vitamin K-dependent carboxylations. This domain is followed by two domains, both of which are homologous to the epidermal growth factor (EGF) precursor. Of said two domains, one has a single hydroxylated aspartic acid residue, i.e. consists of erythro- β -hydroxyaspartic acid. The light and the heavy chains are linked together via one disulfide bond only.

Moreover, protein C requires vitamin K for its normal biosynthesis, and it circulates in blood plasma at a concentration of approximately 4 mg/l.

Protein C is an important regulator of blood coagulation, and it is activated by thrombin complexed with thrombomodulin. This activation involves removal of an activation peptide with 12 amino acids from the amino-terminal end of the heavy chain of protein C, whereby APC is formed. APC is a typical serine proteinase with high arginyl bond specificity.

The substrates degraded by APC are the cofactors known as factor V/V_a and factor VIII/VIII_a, respectively, where "a" denotes the active form of the cofactor. In coagulation pathways, the factor VIII_a forms a membrane-bound complex with the factor IX_a, whereby the coagulation factor X is activated to a serine proteinase denoted coagulation factor X_a. Furthermore, the factor V_a forms a membrane-bound complex with the coagulation factor X_a, whereby prothrombin is activated to thrombin.

The degradation of the factors V_a and VIII_a by APC requires two cofactors. These are protein S, which is a vitamin K-dependent protein, and factor V, which is the unactivated form of factor V_a. The degradation results in cleavage of the factors V_a and VIII_a, whereby their affinity for factors X_a and IX_a, respectively, is reduced. Thus, said cleavage results in a reduction of the rate of formation of both thrombin and coagulation factor X_a. In other words, the blood coagulation cascade is turned off.

As other serine proteinases, APC has its inhibitors. These are protein C inhibitor (hereinafter denoted PCI; plasma concentration approximately 4 mg/l), α_1 -proteinase inhibitor (hereinafter denoted α_1 -antitrypsin; plasma concentration approximately 1 g/l) and α_2 -macroglobulin (plasma concentration approximately 2 g/l). PCI and α_1 -antitrypsin belong to a group of inhibitors sometimes referred to as serpines (serine proteinase inhibitors), and form 1:1 complexes with APC.

The complex formation between APC and PCI proceeds at a slow rate, and it proceeds even slower in respect of α_1 -antitrypsin. This slow rate is manifested by a long half-life for APC in plasma, wherein $t_{1/2}$ is about 20 min.

5 However, the rate of complex formation between APC and PCI is increased by heparin.

Upon complex formation with APC, the serpin is cleaved in its so-called bait region, whereby a stable intermediate acyl complex is formed. With time, the intermediate acyl complex dissociates, whereby APC is re-generated and a proteolytically modified, i.e. cleaved, inactive serine proteinase inhibitor is formed.

The intermediate acyl complexes are removed rather rapidly from the circulatory system. $T_{1/2}$ in baboons is approximately 40 and 140 min for the APC:PCI and APC: α_1 -antitrypsin complexes, respectively. PCI appears to be the prime inhibitor for APC with α_1 -antitrypsin and α_2 -macroglobulin taking over as the PCI concentration is substantially lowered.

20 PCI also inhibits thrombin and factor X_a , both of which are blood coagulation proteinases. In addition, PCI inhibits trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a , prostatic specific antigen (PSA) and the prostata specific kallikrein-like serine proteinase denoted HGK1.

The biological importance of APC can be illustrated in several ways, e.g.

- 30 i) newborn children with homozygous protein C deficiency die during the first days of life in a clinical condition denoted *purpura fulminans*, which is the result of widespread thrombotisation of capillaries;
- ii) heterozygosity for protein C deficiency has in many cases been associated with a thrombophilia that accounts for approximately 2% of all hereditary thrombophilia;

- iii) a frequently occurring (present in 2-10% of caucasian populations) point mutation in factor V, rendering factor V/V_a resistant to degradation by APC, is associated with a mild form of hereditary thrombophilia in the heterozygous form and a more severe form of thrombophilia in the homozygous form;
- iv) disseminated intravascular coagulation is associated with a depletion of activated protein C, a process which often leads to a fatal outcome;
- v) gram negative septicemia (caused by e.g. meningococci) leads to a depletion of activated protein C, a condition shown to be fatal in baboons as well as in humans.

In summary, the considerable biological importance of serine proteinases, particularly protein C, as regulators of blood coagulation as well as their role as key proteins in the so-called protein C anticoagulant pathway has resulted in a great need in the art to develop powerful research tools and extract clinically relevant information from e.g. the concentration of APC in plasma.

In particular, such clinically relevant information could be very useful in the diagnosis of e.g. venous or arterial thrombosis, including coronary infarction or pulmonary embolism.

Prior Art

To serve the above-mentioned purposes and measure the activation of anticoagulant systems involving protein C, numerous methods have been developed, as set forth below.

In Bauer, K.A., Kass, B.L., Beeler, D.L., Rosenberg, R.D., *J. Clin. Invest.*, **74**:2033-2041 (1984), a method is disclosed, wherein the plasma concentration of the activation peptide removed from protein C (*vide supra*) is measured. Since said activation peptide has a very high renal clearance, its plasma concentration is low. Thus,

this method is cumbersome as it requires large amounts of plasma.

In Gruber, A., Griffin, J.H., *Blood*, **79**:2340-2348 (1992), a method is disclosed, wherein the plasma concentration of APC is measured. Here, an ELISA technique with a catching monoclonal antibody against protein C was used. Since said catching monoclonal antibody does not inhibit the activity of APC, an amidolytic assay was used. However, due to the low plasma concentration of APC, the incubation time was generally very long (up to days) and impractical.

In an approach disclosed in Espana, F., Zuazu, I., Vicente, V., Estelles, A., Marco, P., Aznar, J., *Thromb. Haemost.*, **75**:56-61 (1996), heparin-mediated enhancement of the rate of complex formation between APC and PCI is utilised. This method requires the presence of e.g. heparin, benzamidine and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPAC).

However, the most relevant approaches disclosed relate to methods, wherein the concentration of a complex between APC and PCI, or between APC and α_1 -antitrypsin, is measured by use of monoclonal antibodies. These approaches are all based on the assumption that said concentration reflects the concentration of APC, and hence also the degree of activation of the protein C anticoagulant system. Here, a catching monoclonal antibody raised against either APC or PCI is used together with a suitable tracer agent in an ELISA technique.

Thus, if the catching monoclonal antibody is specific for PCI, the tracer agent could be an antibody specific for an epitope in protein C, or vice versa. However, an inherent problem in this approach is that the concentration of free, uncomplexed PCI and APC, respectively, is very high in comparison with the APC:PCI complex to be measured. As an example, due to competitive binding between free, uncomplexed PCI and complex-bound PCI to a catching monoclonal antibody raised against PCI, the

sensitivity of such an approach is greatly reduced. Typical such approaches are disclosed in JP 02-236 452, Espana, F., Griffin, J.H., *Thromb. Res.*, **55**:671-682 (1989) and Espana, F., Vicente, V., Scharrer, I.,
5 Tabernero, D., Griffin, J.H., *Thromb. Res.*, **59**:593-608 (1990).

In an approach disclosed in Minamikawa, K., Wada, H., Wakita, Y., Ohiwa, M., Tanigawa, M., Deguchi, K., Hiraoka, N., Huzioka, H., Nishioka, J., Hayashi, T.,
10 *Thromb. Haemost.*, **71**:192-194 (1994), a precipitated barium salt is used to bind PCI in complex with APC, whereas uncomplexed PCI does not bind to the precipitated barium salt. After centrifugation and dissolution in EDTA (aq), the sample is subjected to ELISA, wherein a
15 catching monoclonal antibody raised against PCI is used. Although useful from a sensitivity point of view, this approach is time-consuming as well as laborious.

In Laurell, L., Carlson, T.H., Stenflo, J., *Thromb. Haemost.*, **60**:334-339 (1988), it is disclosed monoclonal
20 antibodies raised against cleaved PCI, wherein said monoclonal antibodies are applied in an assay for measuring the plasma concentration of APC:PCI complexes. All of said monoclonal antibodies also had specific affinity for uncleaved, uncomplexed PCI.

25 In WO 9822509, there is disclosed monoclonal antibodies which bind specifically to the PSA-ACT complex without significant cross-reactivity with PSA, ACT or CG-ACT. These antibodies are not suitable for monitoring the activity of systems involving protein C inhibitor.

30 In summary, there is still a great need in the art for a monoclonal antibody with high specific affinity for a serine proteinase inhibitor, e.g. PCI, in complex with a serine proteinase, e.g. APC, where said monoclonal antibody has substantially no affinity for PCI in its un-
35 complexed form.

Disclosure of the Invention

According to the invention, there is now provided a new monoclonal antibody which overcomes the problems in the art referred to above. The monoclonal antibody according to the invention is suitable for monitoring the activity of systems involving protein C inhibitor, such as anticoagulant systems, and said monoclonal antibody is characterised by having specific affinity for both

- i) a complex between a serine proteinase and an inhibitor thereof, and
- ii) a cleaved and uncomplexed form of said inhibitor,

while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or being a derivative thereof having the same biological activity.

The monoclonal antibody according to the present invention is obtainable by immunisation of an animal with a mixture of

- i) a complex between a serine proteinase and an inhibitor thereof, and
- ii) a cleaved form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody. Said animal is preferably a mouse, most preferably a Balb/c mouse.

Preferably, said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

As said inhibitor, either protein C inhibitor (PCI) or α_1 -antitrypsin is preferred.

In the most preferred embodiment, the monoclonal antibody according to the invention is obtainable, or obtained, by immunisation with a mixture of cleaved PCI and PCI in complex with PSA.

In another aspect, the present invention also relates to a method for preparation of a monoclonal antibody as defined above, wherein an animal is immunised with a mixture of

- 5 i) a complex between a serine proteinase and an inhibitor thereof, and
 ii) a cleaved and uncomplexed form of said inhibitor,

10 followed by screening for and isolation of said monoclonal antibody.

In yet another aspect, the present invention relates to a method for monitoring the activity of anticoagulant systems involving protein C, wherein a monoclonal antibody as defined above is used in an immunoassay.

15 Preferably, said immunoassay comprises a sandwich-type immunoassay. More preferably, said sandwich-type immunoassay is a DELPHIA[®], ELISA or magnetic bead technique comprising a tracer agent and said monoclonal antibody bound to a surface.

20 In an embodiment of said method, said tracer agent comprises an antibody having specific affinity for said serine proteinase or an epitope shared by said serine proteinase and said inhibitor.

25 In another embodiment, said tracer agent is conjugated to a suitable enzyme and/or labelled with a tracing substance. Said enzyme is e.g. an alkaline phosphatase, horse radish peroxidase or a β -galactosidase. Said tracing substance is e.g. ¹²⁵I, ¹³¹I, Eu³⁺ or Sm³⁺ or a similar lanthanide.

30 In a further aspect, the present invention also relates to a method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monoclonal antibody as defined above is utilised.

35 Furthermore, this invention relates to a method for diagnosis of venous thrombosis, arterial thrombosis, em-

bolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monitoring method as defined above is utilised.

Moreover, the present invention relates to the use
5 of a monoclonal antibody as defined above for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

Also, this invention concerns the use of a monitoring
10 toring method as defined above for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants. In yet another aspect, the present invention relates to a kit for
15 qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody as defined above.

Thus, the present invention allows for precise quantitative measurements of complexes between e.g. human APC
20 and PCI in blood plasma. Indeed, the present invention encompasses measurements of complexes between PCI and thrombin or any other serine proteinase known to form complexes with PCI, e.g. coagulation factor X_a (this embodiment requires the exchange of the tracer agent only).

25 Some of the incentives for the use of a mixture of cleaved and complexed serine proteinase inhibitor for immunisation were gathered from mainly three sources. Firstly, it has been reported that certain monoclonal antibodies against the serpins antithrombin and C-1
30 esterase inhibitor (see e.g. de Agostini, A., Patston, P.A., Marottoli, V., Carrel, S., Harpel, P.C., Schapira, M., *J. Clin. Invest.*, **82**:700-705 (1988) and Asakura, S., Matsuda, M., Yoshida, N., Terukina, S., Kihara, H., *J. Biol. Chem.*, **264**:13736-13739 (1989)) are more or less
35 selective for the complexed form of the inhibitor, albeit no such antibodies have been identified against PCI. Secondly, it has been disclosed in e.g. Björk, I.,

Nordling, K., Olson, S.T., *Biochemistry*, **32**:6501-6505 (1993) that thrombin:antithrombin complexes and anti-thrombin cleaved from such complexes share epitopes. These epitopes are not present on native antithrombin.

5 Thirdly, it has been reported by e.g. Espana, F. and Griffin, J.H. (*vide supra*) that human seminal plasma has a PCI concentration that is about 50-fold higher in comparison with its blood plasma concentration. However, the PCI present in human seminal plasma is unable to form
10 complexes with APC. Indeed, the PCI present in human seminal plasma is the cleaved form of PCI.

The monoclonal antibody according to the present invention, when used as catching antibody in e.g. a sandwich assay, has very high specific affinity for PCI
15 in complex with APC as well as for the proteolytically cleaved PCI, but has substantially no or very low specific affinity for native PCI, viz PCI in its uncleaved and uncomplexed form. In other words, the native PCI has no influence on the binding of APC:PCI complexes to a surface having the monoclonal antibody according to the pre-
20 sent invention bound thereto. Thus, there is substantially no competitive binding of the native PCI to said surface, whereby the sensitivity of the method according to the invention is high.

25 The method according to the present invention has been devised such that it can easily be automated and adopted for use in modern automated laboratory instrumentation using e.g. magnetic beads coated with the monoclonal antibody of the invention.

30 The present invention is applicable both as a powerful research tool and in conjunction with diagnosis of e.g. venous thrombosis, embolism and coronary infarction. In particular, the latter application could be very useful as it may detect coronary infarction much earlier
35 than existing methods, most of which measure the concentration of enzymes or other proteins released from the damaged myocardium (CK-MB, troponin T or myoglobin). The

currently used methods are not diagnostically helpful until coronary ischemia has already damaged the myocardium. Detectable levels of CK-MB and troponin T are usually not present until 3-5 h (2-3 h for myoglobin) after the infarction, depending on its size.

Furthermore, the present invention may also help in the differentiation between coronary infarction and unstable angina, thereby improving current diagnostic procedures most significantly.

In a sandwich-type immunoassay, such as a DELPHIA[®] technique, two monoclonal antibodies are used. According to a preferred embodiment of the present invention, one has specific affinity for PCI, and this monoclonal antibody is bound to a surface and denoted "catcher" in a DELPHIA[®] context. The other monoclonal antibody has specific affinity against protein C and is denoted "tracer agent" in a DELPHIA[®] context. According to the present invention, the catcher has been carefully selected to circumvent the problems associated with the prior art (*vide supra*), i.e. that the large molar excess (normally more than 1000-fold) of uncomplexed protein, be it protein C or PCI, competes with the complexes to be measured by binding to a surface-bound monoclonal antibody.

Thus, in a preferred embodiment, the inventor has generated mouse monoclonal antibodies which have very high specific affinity for complexed PCI and cleaved PCI, but substantially no affinity for native PCI, viz PCI in its uncleaved and uncomplexed form. Such monoclonal antibodies are used as catcher in an immunoassay, as set forth below, and they were obtained by immunising Balb/c mice with a mixture of cleaved PCI and PCI in complex with a serine proteinase known as prostatic specific antigen (PSA). Furthermore, the inventor has devised approaches for carefully selecting, i.e. screening and isolating, such monoclonal antibodies, as is disclosed hereinbelow.

The present invention is further illustrated by the following non-limiting examples together with the accompanying figures.

Description of the figures

5 Fig. 1 shows affinity chromatograms for APC-complexed (A), cleaved (B) and native (C) PCI, respectively, obtained on an Affi Gel[®] 10 column (0,5 x 10 cm), onto which the monoclonal antibody M36 was immobilised. In each one of the samples A, B and C, 30 µg of the respective PCI analyte was chromatographed. The column was
10 equilibrated with 50 mM Tris-HCl/0,5 M NaCl (pH 7,5). The flow rate was 0,1 ml/min. Bound protein was eluted with 0,1 M glycine-HCl/0,5 M NaCl with pH 2,7 (indicated by the arrow). The continuous line represents absorbance,
15 whereas the "o-o"-line represents fluorescence. The early eluting peak in A consists of UV absorbing low molecular weight compounds and APC from cleaved complexes.

Fig. 2 shows measurements in real time of the interaction of APC:PCI complexes and cleaved PCI with the
20 monoclonal antibody M36 using the surface plasmon resonance (SPR) technique. A) illustrates the interaction of APC:PCI complexes with M36, and the concentrations of the APC:PCI complexes were 17,6 nM (●), 8,8 nM (◆), 4,4 nM (▲) and 2,2 nM (▼). B) illustrates the interaction of
25 cleaved PCI with M36, and the concentrations of cleaved PCI were 38,5 nM (●), 7,7 nM (▲), 3,9 nM (▼), 1,9 nM (■) and 1,0 nM (◆). The lines were fitted to the experimental data, some of which are denoted by the symbols.

Fig. 3 depicts dose-response curves for measurements
30 of APC:PCI complexes. Each point represents the mean value of duplicate measurements. (◆) illustrates APC:PCI complexes in buffer, whereas (Δ) illustrates APC:PCI complexes in citrated plasma to which benzamidine has been added to a final concentration of 50 mM. The signal given
35 by APC:PCI complexes prior to the addition of standard has been subtracted.

Examples

Preparation of proteins:

Native PCI was purified from human plasma according to a method described in Laurell, M. et al. (*vide supra*).

5 Cleaved PCI and PCI in complex with PSA were purified from human seminal plasma using the same method.

Protein C was purified from human plasma by adsorption to barium citrate, elution with EDTA, column chromatography on DEAE Sepharose® and use of an im-
10 mobilised monoclonal antibody against protein C (HPC-4) that recognises a calcium-dependent epitope in the N-terminal EGF-like domain, as disclosed in Ohlin, A.K., Stenflo, J., *J. Biol. Chem.*, **262**:13798-13804 (1987). Protein C was eluted from the column with 0,1 M sodium
15 acetate/acetic acid buffer containing 0,5 M NaCl (pH 4,0). By addition of concentrated Tris buffer, an immediate adjustment to pH 7,5 was performed.

APC was prepared by thrombin-mediated activation of protein C, followed by isolation of APC by chromatography
20 on an HPC-4 column, as described by Ohlin, A.K. and Stenflo, J. (*vide supra*).

Complexes used as standard in the method of measuring APC:PCI complexes were made by incubation of APC with a two-fold molar excess of PCI in 50 mM Tris-HCl,
25 0,1 M NaCl, 1 mM EDTA with pH 7,5. The complexes were isolated by affinity chromatography on an HPC-4 column, as described above. The purity of the isolated complex was established by SDS-PAGE and silver staining of the gel. The concentration of the complex in a standard
30 solution was determined by measurement of Gla, assuming the presence of nine Gla residues per protein C molecule. Typically, the standard solution was made 0,1% in bovine serum albumin (BSA) and stored in aliquots at -70°C.

Used methods:

35 In the DELPHIA® assay, microtiter plates (Fluor Maxisorp, Nunc) coated with the monoclonal antibody M52 (1 µg/100 µl per well) and incubated overnight at +4°C

were washed with a wash solution (Wallac) and blocked with 0,7% BSA in an assay buffer (Wallac) for 2 h. After washing of the plates, 100 μ l of each fraction, with a dilution of 1:1000 in assay buffer, was added to the wells. The plates were incubated for 1 h on a DELPHIA[®] plate shaker (Wallac) and then washed. Eu³⁺ labelled monoclonal antibodies M11-5 (B and C in figure 1) or HPC-4 (A in Fig. 1), having a dilution in assay buffer to a concentration of 20 ng/100 μ l, were added, followed by 1 h incubation on the shaker. After washing, 200 μ l enhancement solution (Wallac) was added, followed by 5 min incubation on the shaker. Fluorescence was then recorded in a DELPHIA[®] fluorimeter (Wallac).

Surface plasmon resonance (SPR) was measured with the BIAcore[®] technology (Pharmacia). Streptavidin was immobilised on the BIAcore[®] sensorchip CM5 according to the instructions of the manufacturer. The immobilised streptavidin gave between 5400 and 6800 response units (RU). Biotinylated antibody, having a concentration of 0,34 mg/ml in 10 mM Tris-HCl/150 mM NaCl (pH 7,75), was immobilised at a flow rate of 5 μ l/min, which resulted in a response of 2800-3000 RU. Aliquots of protein stock solution were diluted in flow buffer and 20 μ l was injected during the association phase at a constant flow rate of 5 μ l/min. The dissociation phase was monitored at the same flow rate for 10 min. The system was regenerated by short pulses of 50 mM NaOH at a flow rate of 2 μ l/min. For the fitting of the lines to the experimental data, see e.g. He, X., Shen, L., Malmberg, A.C., Smith, K.J., Dahlback, B., Linse, S., *Biochemistry*, **36**:3745-3754 (1997).

Production of monoclonal antibodies:

Balb/c mice were immunised with a mixture of PCI in complex with PSA (approximately 80%) and PCI cleaved from such a complex (approximately 20%). Each mouse was immunised three times with approximately 10 μ g of said mixture each time. The two first immunisations were made in-

tracutaneously with said mixture emulsified in Freund's complete adjuvant, whereas the third immunisation was administered subcutaneously with Freund's incomplete adjuvant. Testing of the mouse plasma, using an ELISA with an appropriate antigen coated in a 96 well microtiter plate, indicated a good antibody response after about two months. Five, four and three days prior to cell fusion, the mice were injected intraperitoneally with approximately 50 µg of the same immunogen (no adjuvant) each time. On cell fusion, the spleen cells were extracted and fused with the myeloma cell line SP2/-Agl4 using 45% polyethylene glycol 1540 and 7% DMSO (dimethyl sulphoxide) under standard conditions (see Borrebäck, C.A.K., Eylar, M.E., *J. Biol. Chem.*, **256**:4723-4725 (1981)). Fused cells in DMEM medium supplemented with HAT (hypoxanthine, aminopterin, thymidine) were seeded into 96 well microtiter plates at a cell density of approximately 10^5 cells per well together with approximately 2×10^4 feeder cells per well. After about ten days, hybridoma supernatants were screened for antibody production.

Clones producing antibodies with interesting properties were identified as described below. The hybridomas were subcloned twice by limiting dilution using 96 well microtiter plates (0,5 to 1 cell per well) using mouse peritoneal macrophages as feeder cells. Stable clones producing monoclonal antibodies of interest were grown to high cell density and injected intraperitoneally into pristane-primed (0,2 ml per mouse) mice for antibody production. Antibodies were also produced on preparative scale by tissue culture (Technomouse®, Integra Biosciences). The antibody was purified from the ascitic fluid/tissue culture medium using chromatography on Protein A Sepharose® according to the instructions of the manufacturer. The cell lines were preserved frozen in 95% calf serum and 5% DMSO in liquid nitrogen.

Procedures for the identification of antibody producing clones that were specific for the complexed/-

cleaved form of PCI were carefully tailored. Microtiter plates were coated overnight with rabbit antimouse immunoglobulins (DAKO; 1 µg/well in 50 µl) in 0,1 M carbonate buffer with pH 9,6. After washing, the wells were

5 "blocked" for 15 min with a solution of bovine serum albumin (10 mg/ml, 100 µl per well) in 50 mM Tris-HCl, 0,1 M NaCl with pH 7,4. After washing, culture medium was added to identify antibody producing clones. After washing again, the wells were incubated with a tracer agent

10 in the above buffer for 1 h. As ¹²⁵I-labelled tracer agents, native PCI as well as APC:PCI complexes were used. The clones were also tested with the same method and found not to react with APC. After 1 h of incubation with the tracer agent, the plates were washed with the

15 buffer, after which the radioactivity was measured. By having APC in the complex labelled with ¹²⁵I, it was possible to select antibody producing clones that bound PCI in complex with APC. Moreover, a comparison with the binding of labelled, native PCI helped to identify clones

20 that bound PCI in complex with APC with high specific affinity, but had a low specific affinity for native PCI.

One of the antibodies (M36) with specific affinity for APC:PCI complexes and cleaved PCI was carefully characterised. Three other monoclonal antibodies were

25 also used; one against protein C that has been characterised (Ohlin, A.K. and Stenflo, J., *vide supra*), and two against PCI which do not discriminate between cleaved and native PCI. One of the latter two antibodies has been described before (M11-5; Laurell, M., Christensson, A.,

30 Abrahamsson, P-A., Stenflo, J., Lilja, H., J. Clin. Invest., **89**:1094-1101 (1992)), whereas the other one (M52) was new (preparation disclosed herein).

Characterisation of monoclonal antibodies:

Several monoclonal antibodies with the appropriate

35 properties were produced. Of these, M36 was carefully characterised. This monoclonal antibody was immobilised on an Affi Gel[®] 10 column (Biorad) and APC-complexed (A),

cleaved (B) and native (C) PCI were chromatographed on the column, as is shown in Fig. 1. The antibody bound to both cleaved and complexed PCI with high affinity, but was found not to bind or even retard the native PCI.

5 The dissociation constants of the antibody for its binding to PCI in complex with APC, cleaved PCI and native PCI, respectively, were measured with standard plasmon resonance technique. The dissociation constant, Kd, of M36 was 4×10^{-10} M for PCI in complex with APC and
10 2×10^{-10} M for the cleaved PCI, respectively, as is shown in Fig. 2. The value of Kd for the native PCI was too low, i.e. $\geq 10^{-5}$ M, to be determined accurately (not shown in Fig. 2). In summary, these results established that the selected monoclonal antibody M36 binds complexed PCI
15 and cleaved PCI with very high affinity, but has very low, if any, affinity for native PCI.

As a tracer agent, a monoclonal antibody against protein C was used. This antibody, which was made by fusion of spleen cells with the myeloma cell line NS 1,
20 is described by Ohlin, A.K. and Stenflo, J. (*vide supra*). Its epitope is in the calcium-binding N-terminal EGF-like domain of protein C, a domain exposed on both protein C and APC. Furthermore, this antibody binds to protein C and APC with high affinity, but the dissociation constant
25 has not been quantitatively measured. The antibody binds to said epitope in a calcium-dependent manner, and it should be stated that no special requirements are made on this antibody, except that it should bind complexed APC with high affinity and can be labelled without any loss
30 of affinity for the antigen. Among the protein C antibodies tested, this one worked best.

Collection of blood:

Blood was collected in 5 ml siliconised glass vacuum tubes (Becton Dickinson) containing 0,5 ml of a 0,129 M
35 trisodium citrate solution, to which benzamidine had been added to a concentration of 50 mM. Usually, the samples were centrifuged within 4 h after the blood was collec-

ted, and the plasma was either assayed immediately or frozen at -70°C . The benzamidine greatly reduces the rate of complex formation between PCI and proteinases, such as kallikreine.

5 Performance of the assay:

 The catching antibody (M36) was biotinylated with a N-hydroxysuccinimide derivative of biotin (NHS-LC-Biotin[®]; Pierce) according to the instructions of the manufacturer. The biotinylated protein was dialysed against a
10 0,1 M sodium fosfate buffer (pH 7,0) containing 0,1% NaN_3 , and it was typically stored at a concentration of 0,89 mg/ml at $+4^{\circ}\text{C}$.

 To avoid the use of radioactivity and the need for enzyme conjugation of the tracer agent, i.e. HPC-4, a so-called DELPHIA[®] method was used. In said method, an Eu^{3+}
15 labelled tracer agent is utilised, and in this assay, the DELPHIA[®] Eu-labelling kit 1244-302 (Wallac) was used. The HPC-4 antibody can be heavily labelled with up to 80 Eu^{3+} ions per antibody molecule without any loss of affinity
20 for protein C. The tracer agent was stored as frozen aliquots at -20°C at a concentration of 0,043 mg/ml. Even if the DELPHIA[®] procedure was used in the present investigation, any commonly used means of labelling the tracer agent can probably be used.

25 The biotinylated M36 antibody was diluted with assay buffer (Wallac assay buffer: 50 mM Tris-HCl, 0,9% NaCl, 0,05% NaN_3 , 0,01% Tween 40, 0,05% bovine immunoglobulin, 0,5 μM DPTA and 20 $\mu\text{g}/\text{ml}$ cherry red) to a concentration of 2 $\mu\text{g}/\text{ml}$ prior to use. The samples were analysed in
30 duplicate. 50 μl of buffer blank, standard samples, control samples, plasma blanks and plasma (patient) samples, respectively, were added to the wells of a 96 well microtiter plate (Sero-Wel, Bibby Sterlin Ltd.) followed by addition of 50 μl of the biotinylated M36 antibody to the
35 standard, control and plasma samples, whereas 50 μl of assay buffer was added to the other samples. The plates were then put on a LKB, Wallac shaking device for 30 s

followed by incubation at room temperature for 80 min. The entire sample was then transferred to a streptavidine coated plate (Microtitration strips, DELPHIA[®], Wallac), after which the plate was shaken for 60 min on the shaking device, followed by washing in a plate washer (LKB, Wallac). 100 μ l of the tracer agent solution was then added (20 ng/100 μ l per well, diluted in assay buffer), followed by incubation on the shaking device for another 40 min at room temperature. After washing (Wallac wash fluid; Tris buffer, salt and Tween 20, pH 7,8), 200 μ l of an enhancer solution (Wallac enhancement solution) was added to each well, and the plate was shaken on the shaking device for 10 min. Fluorescence was determined in an LKB-Wallac fluorimeter, and the APC:PCI complex formation was calculated by use of the program MultiCalc (Wallac). Here, it should be emphasized that the method according to the present invention gives a linear dose-response curve, as expected for a very high affinity antibody in a sandwich-type assay (figure 3).

In an alternative to the above procedure, the samples can be assayed directly on the streptavidine coated plate together with a biotinylated antibody, thereby avoiding the first incubation in a 96 well microtiter plate. In this manner, an even quicker procedure could be achieved without loss of performance. The time for the incubation with tracer can also be reduced. Such an alteration does not lead to any loss in the performance of the assay.

It should be noted that the standard curves are identical when made in buffer and in plasma, thus corroborating that the uncleaved inhibitor does not influence the assay. The signal given by APC:PCI complexes prior to the addition of standard has been subtracted (Fig. 3).

CLAIMS

1. A monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, said
5 monoclonal antibody having specific affinity for both

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said inhibitor,

10 while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or a derivative thereof having the same biological activity.

2. A monoclonal antibody according to claim 1, wherein said monoclonal antibody is obtainable by
15 immunisation of an animal with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said
20 inhibitor,

followed by screening for and isolation of said monoclonal antibody.

3. A monoclonal antibody according to claim 2, wherein said animal is a mouse, preferably a Balb/c
25 mouse.

4. A monoclonal antibody according to any one of the preceding claims, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymo-
30 trypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

5. A monoclonal antibody according to any one of the preceding claims, wherein said inhibitor is protein C inhibitor (PCI) or α_1 -antitrypsin.
35

6. A method for preparation of a monoclonal antibody as defined in any one of claims 1-5, wherein an animal is immunised with a mixture of

5 i) a complex between a serine proteinase and
 an inhibitor thereof, and

 ii) a cleaved form of said inhibitor,
followed by screening for and isolation of said monoclonal antibody.

7. A method for preparation of a monoclonal antibody
10 according to claim 6, wherein said animal is a mouse, preferably a Balb/c mouse.

8. A method for monitoring the activity of systems involving protein C inhibitor, wherein a monoclonal antibody as defined in any one of claims 1-5 is used in an
15 immunoassay.

9. A method according to claim 8, wherein said immunoassay comprises a sandwich-type immunoassay.

10. A method according to claim 9, wherein said sandwich-type immunoassay is a technique comprising a
20 tracer agent and said monoclonal antibody bound to a surface.

11. A method according to claim 10, wherein said tracer agent comprises an antibody having specific affinity for said serine proteinase or an epitope shared
25 by said serine proteinase and said inhibitor.

12. A method according to claim 11, wherein said tracer agent is conjugated to a suitable enzyme and/or labelled with a tracing substance.

13. A method according to claim 12, wherein said
30 enzyme is an alkaline phosphatase, horse radish peroxidase or a β -galactosidase.

14. A method according to claim 13, wherein said tracing substance is ^{125}I , ^{131}I , Eu^{3+} or Sm^{3+} or a similar lanthanide.

35 15. A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders

involving lupus anticoagulants, wherein a monoclonal antibody according to any one of claims 1-5 is utilised.

16. A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, 5 disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to any one of claims 8-14 is utilised.

17. Use of a monoclonal antibody according to any one of claims 1-5 for in vitro diagnosis of venous throm- 10 bosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

18. Use of a method according to any one of claims 8-14 for in vitro diagnosis of venous thrombosis, ar- 15 terial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

19. A kit for qualitative or quantitative determination of the activity of systems involving protein C 20 inhibitor comprising a monoclonal antibody according to any one of claims 1-5.

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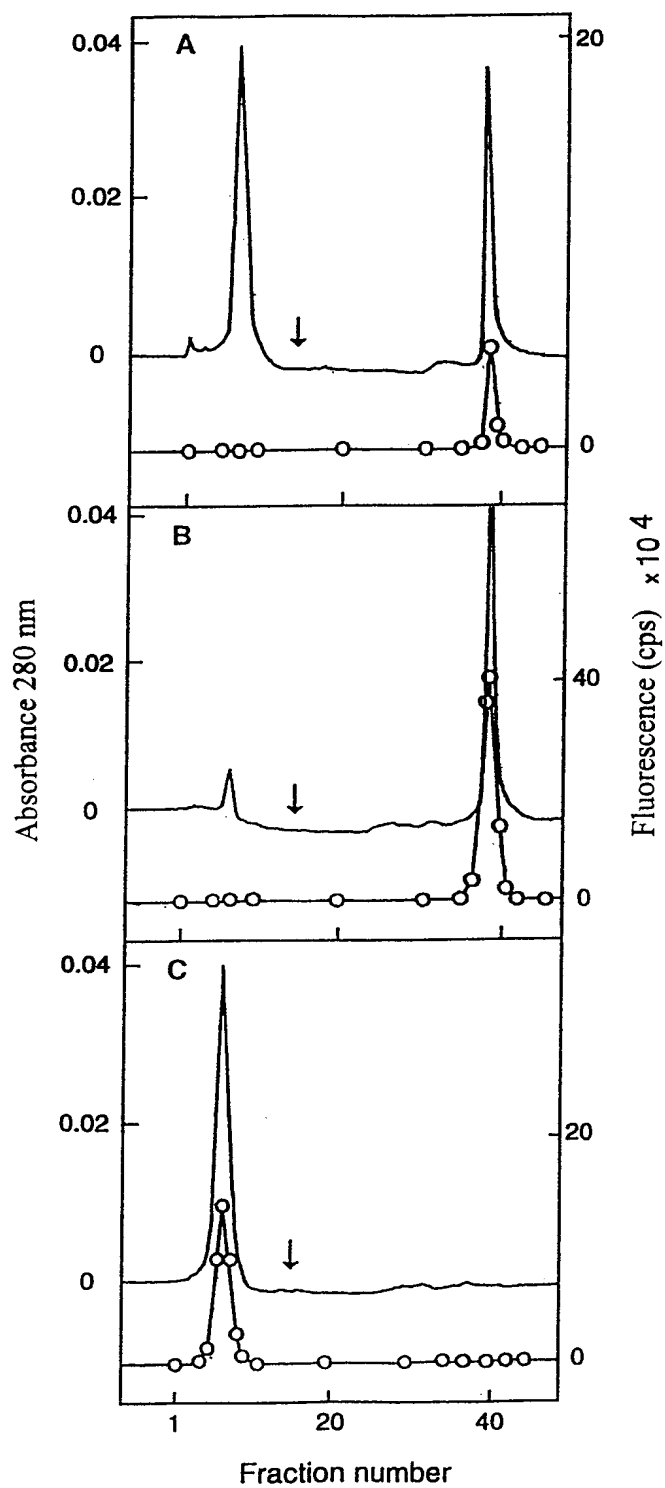


Figure 1

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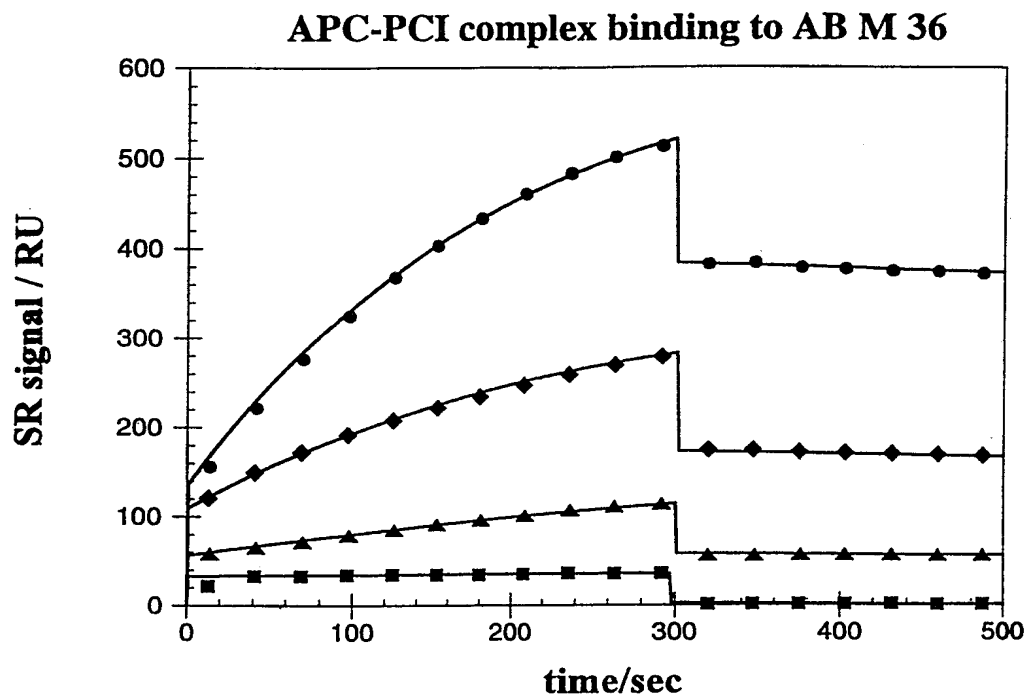


Figure 2A

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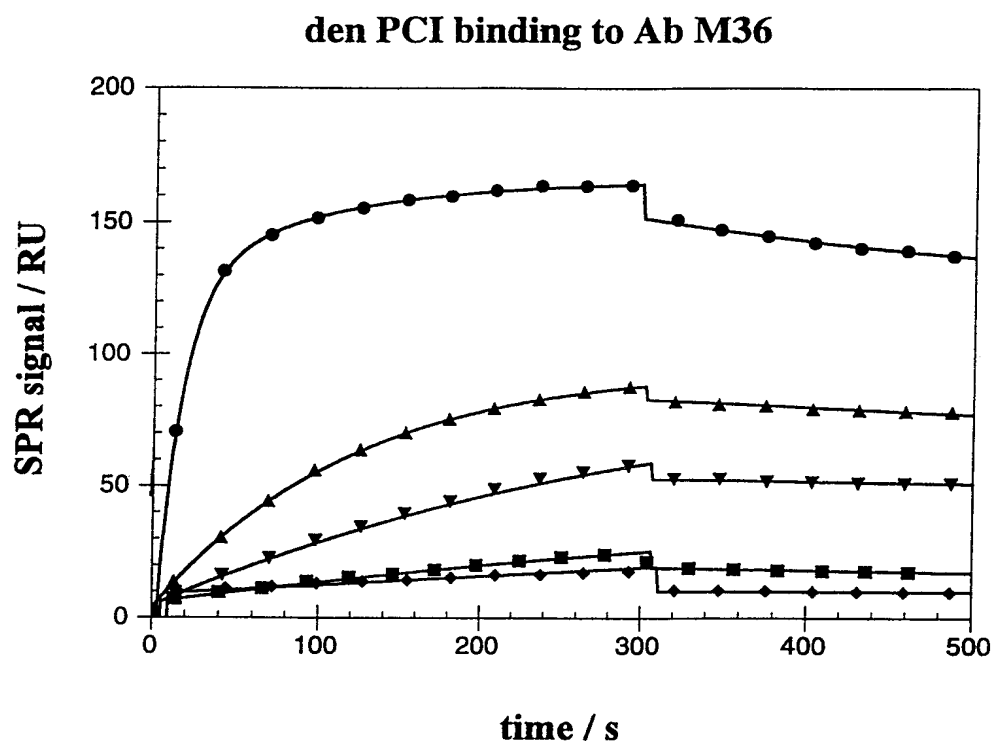


Figure 2B

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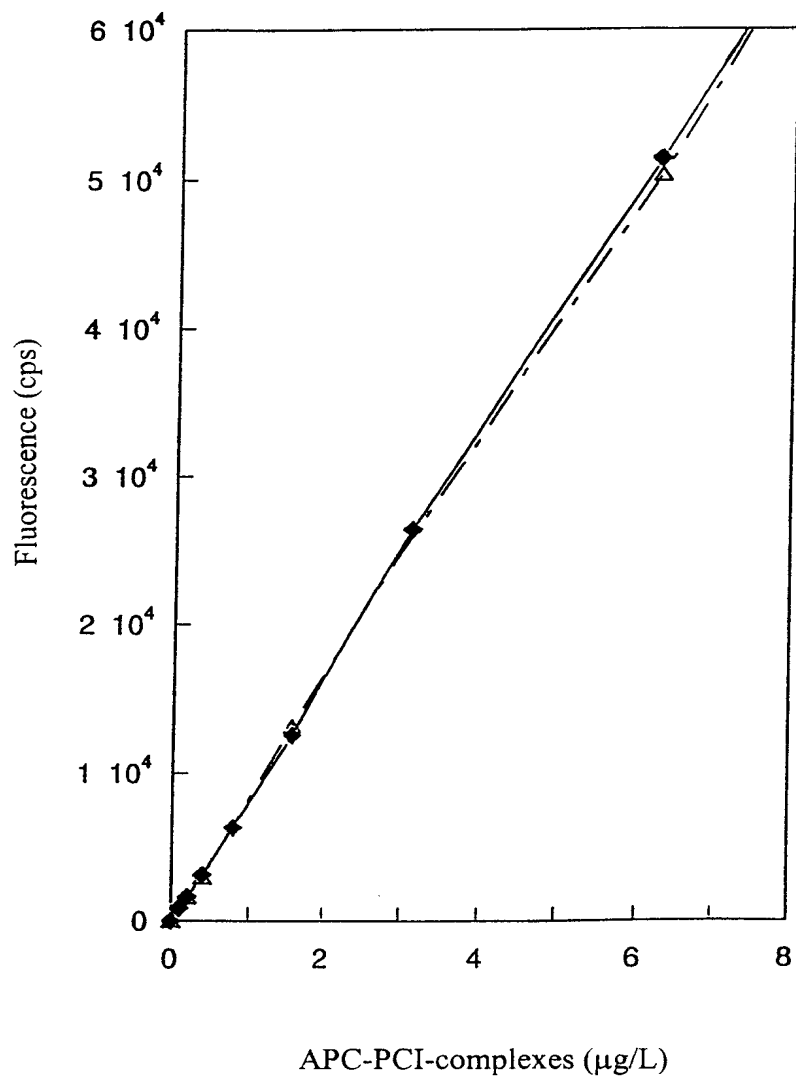


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/00210

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 16/38, G01N 33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	File WPI, Derwent accession no. 99-341640, EISAI CO LTD: "New monoclonal antibody - useful for detecting presence of protein C inhibitor and protease complex"; & JP,A1124399, 990511 DW9929 --	1-19
X	WO 9822509 A1 (BECKMAN INSTRUMENTS, INC.), 28 May 1998 (28.05.98), page 6, line 19 - page 7, line 4, the abstract --	1-19

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

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Date of the actual completion of the international search

6 June 2000

Date of mailing of the international search report

14 -06- 2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/00210

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 73, Embase, Dialog accession no. 07174203, Embase accession no. 1998062258, Ruleva N.Y. et al: "Preparation of monoclonal antibodies against human thrombin - antithrombin III complex", Immunologya (IMMUNOLOGYA) (Russian Federation) 1997, -/6 (30-33) --	1-19
X	EP 0669344 A2 (DAIICHI PURE CHEMICALS CO. LTD), 30 August 1995 (30.08.95), page 2, line 40 - line 51 --	1-19
A	Dialog Information Services, File 155, Medline, Dialog accession no. 5441215, Medline accession no. 89114718, Laurell M. et al: "Monoclonal antibodies against the herparin-dependent protein C inhibitor suitable for inhibitor purification and assay of inhibitor complexes", Thromb Haemost (GERMANY, WEST) Oct 31 1988, 60 (2) p334-9 --	1-19
A	File WPI, Derwent accession no. 90-330685, EISAI CO LTD: "Determining complex of activated human protein C - using anti-human protein C inhibitor monoclonal", & JP,A,2236452, 900919 DW9824 -- -----	1-19

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/SE 00/00210

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